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Multiple Caspases Are Activated after Traumatic Brain Injury: Evidence for Involvement in Functional Outcome

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ABSTRACT

Caspase-3 is a cysteine protease that is strongly implicated in neuronal apoptosis. Activation of caspase-3 may be induced by at least two major initiator pathways: a caspase-8–mediated pathway activated through cell surface death receptors (extrinsic pathway), and a caspase-9–mediated pathway activated by signals from the mitochondria that lead to formation of an apoptosomal complex (intrinsic pathway). In the present studies, we compare the activation of caspases-3, -8, and -9 after lateral fluid-percussion traumatic brain injury (TBI) in rats. Immunoblot analysis identified cleaved forms of caspases-3 and -9, but not caspase-8, at 1, 12, and 48 h after injury. Immunocytochemistry specific for cleaved caspases-3 and -9 revealed their expression primarily in neurons. These caspases were also frequently localized in TUNEL-positive cells, some of which demonstrated morphological features of apoptosis. However, caspases-3 and -9 were also found in neurons that were not TUNEL-positive, and other TUNEL-positive cells did not show activated caspases. In contrast to caspases-3 or -9, caspase-8 expression was only minimally changed by injury. An increase in expression of this caspase was undetectable by immunoblotting methods, and appeared as positive immunostaining restricted to a few cells within the injured cortex. Treatment with the pan-caspase inhibitor z-VAD-fmk at 15 min after TBI improved performance on motor and spatial learning tests. These data suggest that several caspases may be involved in the pathophysiology of TBI and that pan-caspase inhibition strategies may improve neurological outcomes.

Key words: brain injury; caspase; cysteine protease; mitochondria; neuron; trauma

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) initiates physiological and cellular secondary injury responses that ultimately lead to neuronal death and neurological dysfunction. Although necrosis has long been acknowledged as a major cell death mechanism after TBI, a significant role for apoptotic cell death has recently been described (Rink et al., 1995; Yakovlev et al., 1997; Fox et al., 1998; Clark et al., 1999; Newcomb et al., 1999; Ng et al., 2000).

Activation of caspase proteases has been strongly implicated in apoptosis after CNS injuries, including TBI (Yakovlev et al., 1997; Namura et al., 1998; Clark et al., 1999, 2000; Fink et al., 1999; Krajewski et al., 1999; Springer et al., 1999; Beer et al., 2000b; Buki et al., 2000; Li et al., 2000; Lu et al., 2000). Caspases are cysteine proteases that are expressed as inactive pro-forms (zymogens) that, upon activation, are cleaved into large and small subunits that form heterotetramers with enzymatic activity (for caspase review, see Eldadah and Faden,

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2000; Reed, 2000). Caspases have been categorized into three groups, based on function: (1) initiator/apical caspases (caspases -2, -8, -9, -10); (2) executioner/effector caspases (caspases-3, -6, -7); (3) inflammatory caspases (caspases-1, -4, -5, -11). Initiator/apical caspases are so-named because of their relative position upstream of other caspases in putative apoptotic pathways. Executioner/effector caspases primarily function downstream of apical caspases, and directly cleave various substrate proteins responsible for apoptosis. Inflammatory caspases play dual roles as both cell-death proteins and processors of pro-inflammatory cytokines.

To date, two major routes of caspase activation have been described. An extrinsic pathway involves binding of cell surface receptors by specific ligands, that directly trigger caspase activation through adaptor proteins. Thus, apoptosis is induced through signals received at the cell surface. This pathway is utilized by members of the tumor necrosis factor (TNF) superfamily. Examples include binding of TNF receptor I by TNF/lymphotoxin, and binding of Fas by Fas ligand (Chinnaiyan et al., 1995; Juo et al., 1998; Varfolomeev et al., 1998). Either of these events can serve to activate pro-caspase-8, which subsequently activates pro-caspase-3 (Stennicke et al., 1998). In contrast, the intrinsic pathway is triggered by intracellular activation through signals derived from mitochondria. Cytochrome c is released from mitochondria and, in turn, binds the caspase-activating protein Apaf-1, which oligomerizes and binds pro-caspase-9, resulting in its cleavage/activation (Li et al., 1997; Hakem et al., 1998; Saleh et al., 1999; Zou et al., 1999). Subsequently, caspase-9 activates pro-caspase-3 (Slee et al., 1999). Although this classification of extrinsic and intrinsic pathways has proved generally useful, it should be noted that the scheme is somewhat oversimplified, as evidenced by cross-talk and feed-forward amplification of these pathways (Saleh et al., 1999; Zou et al., 1999; Reed, 2000).

Activated caspases cleave a diverse group of substrates, including other enzymes, and proteins involved in cell structure, signal transduction, transcription and DNA repair (reviewed in Eldadah and Faden, 2000). Effector caspases, in particular, cleave proteins that maintain the integrity of nucleic acids (ICAD, PARP) and cell structure (lamins, α-fodrin). Degradation of such proteins leads to membrane blebbing, nuclear fragmentation or condensation, cell shrinkage and/or formation of apoptotic bodies, all of which are morphological hallmarks of apoptotic demise.

Information about caspase activation, and its potential effect on neurological recovery and tissue loss after traumatic brain injury is primarily confined to caspases-1, -2, and -3 (Yakovlev et al., 1997; Beer et al., 2000b; Buki et al., 2000; Clark et al., 2000). Recently, the activation of caspases-8 and -9, was described in models of cerebral ischemia and traumatic spinal cord injury (Krajewski et al., 1999; Springer et al., 1999; Velier et al., 1999; Keane et al., 2001b). As these caspases are initiators of well-described intrinsic and extrinsic apoptotic pathways, respectively, information about them provides particular insight into the type of apoptotic mechanisms that may be important in secondary injury responses. To address this issue, we evaluated the activation of caspase-8, -9, and -3 after TBI induced by the rat lateral fluid-percussion model via temporal immunoblot analysis of cleaved fragments of caspases-3, -8, and-9. In addition, we performed immunocytochemistry utilizing antibodies specific for active caspases, to determine their relative distribution and cell-type specific expression, as well as their association with a late event associated with apoptotic cell death—namely, DNA fragmentation as assessed by the TUNEL assay. Lastly, we evaluated the effect of treatment with a broad-spectrum caspase inhibitor on motor and cognitive function over time after injury.

MATERIALS AND METHODS

Traumatic Brain Injury

The lateral fluid-percussion model of rat brain injury has been previously detailed (McIntosh et al., 1989). Briefly, a craniotomy was performed over the left parietal cortex of anesthetized (70 mg/kg sodium pentobarbital, i.p.), intubated, male Sprague-Dawley rats (400 ± 25 g, Harlan) ventilated on room air. Injury was induced by a brief, pressurized (2.5–2.6 atm), saline pulse delivered through the craniotomy to the intact dura. Sham controls were subjected to identical surgical procedures, with the exception that no fluid pulse was delivered. The caudal artery was cannulated to monitor blood gas and pressure throughout the procedure. Body (rectal) (37.5–38.5°C) and brain temperature (lateralis muscle) (36.5–37.5°C) were assessed and maintained within normal ranges. Three different sets of animals were prepared: (1) for immunoblot and activity assay studies, rats were subjected to the brain injury procedure and then sacrificed either 1, 12, 48, or 168 h later (n = 5–6/group); naive rats were used as controls (n = 5–6); (2) for immunocytochemical studies, rats were subjected to brain injury and then sacrificed either 4 or 24 h later (n = 3/group); controls were sacrificed either 4 or 24 h after sham injury performed as described above (n = 3/group); (3) for the drug treatment study, 25 mM z-VAD-fmk was dissolved in DMSO and injected intracerebroventricularly (5 μL/2 min) at 15 min after injury (n = 12); con-

trols received equi-volume injections of DMSO vehicle ($n = 13$).

All procedures involving live animals were approved by the Georgetown University Animal Care and Use Committee, and were performed according to principles enumerated in the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW publication NIH 85-23-2985).

Preparation of Tissue Homogenates and Immunoblotting

After decapitation, rat cortices were resected on ice and immediately frozen (−70°C). Later, the samples were weighed and homogenized in a 5:1 (wt/v) ratio of RIPA buffer (1% Na deoxycholate, 0.1% SDS, 1% Triton X-100, 0.01 M Tris HCl, pH 8.0, 0.14 M NaCl, 1 mM iodoacetamide, 1 mM AEBSF, 1 mM Aprotinin). Homogenates were centrifuged (10,000 × *g*, 4°C) for 15 min and the resulting supernatent removed and frozen in 50-μL aliquots. One aliquot was used to determine protein concentration via the method of Bradford (Bradford, 1976). Protein lysates were normalized for total protein content (50 μg/lane) and loaded into 16% (caspase-3) or 4–20% (caspases-9 or -8) Tris-glycine gels and electrophoresed at 100 V for 2–3 h in a Tris/glycine/SDS running buffer. Proteins were transferred to PVDF membranes (25 V for 2 h, 4°C) in Tris/glycine buffer. The membrane was blocked for 2 h with 5% dried milk in Tris/borate/1% Tween 20 buffer at room temperature, then incubated overnight with primary polyclonal rabbit antisera. The next day, membranes were washed three times for 10 min each in Tris/borate buffer, and the protein bands were detected by enhanced chemilumenescence-based ECL methods using Hyperfilm (Amersham). The following primary antibodies were used: caspase-3 (Bur1797, 1:2,000; Burnham Institute, La Jolla, CA); caspase-9 (Bur81, 1:2,000); and caspase-8 (SK440, 1:2,000; SmithKline Beecham, King of Prussia, PA). The specificity of these antibodies has been demonstrated previously (Velier et al., 1999; Krajewski et al., 1999), except for Bur81, which was prepared and specificity demonstrated as described below. Recombinant active caspases and/or cells that are known to highly express caspases were used as positive controls. All blots were directly reprobed with anti-β-actin antibody as an internal control for loading and transfer of proteins (Liao et al., 2000). Blots shown are representative of experiments that were repeated on samples taken from three to five different animals/timepoint.

Production and Specificity of Bur81 Antibody

Anti-caspase-9 serum B81 was produced using recombinant human caspase-9 full-length His 6 protein as an immunogen. Recombinant C9/B81 was produced as a fusion protein with a C-terminal his 6 tag. This protein was expressed in BL21 (DE3) cells by induction with 1 mM IPTG. Following cell growth and lysis, the clarified cell lysate was applied to a Ni-NTA column and eluted with an imidazole gradient. The pooled C9 fractions were dialyzed against 50 mM Tris at pH 8.8 and applied to a 10/10 FPLC mono Q column (Pharmacia, Piscataway, NJ) and eluted with a NaCl gradient. New Zealand white female rabbits were injected subcutaneously with a mixture of recombinant protein (0.1–0.25 mg protein per immunization) and 0.5 mL of Freund's complete adjuvant (dose divided over 10 injections sites) and then boosted three times at weekly intervals, followed by another 3–20 boostings at monthly intervals of recombinant protein immunogens in Freund's incomplete adjuvant, before collecting blood and obtaining immune serum. Specificity of the antisera was tested for reactivity to *in vitro* translated caspases as follows. Caspase-3, -6, -7, -8, -9, and -10 cDNAs subcloned into pcDNA-3 (Invitrogen, Carlsbad, CA) were transcribed and translated *in vitro* using 1 μg of plasmid DNA, T7 RNA polymerase and TNT reticulocyte lysates (Promega, Madison, WI), according to the manufacturer's protocol, in the presence of [^{35}S]-labeled L-methionine (~1 mCi/mmole) (Amersham, Piscataway, NJ). The *in vitro* translated caspase proteins were then subjected to SDS-PAGE/immunoblot analysis using Bur81 antisera.

Immunocytochemistry

Animals were anesthetized and intracardially perfused with saline and 4% paraformaldehyde at selected times after injury. Brains were removed, protected in sucrose, frozen in O.C.T. media, sectioned serially (40 μm), and stored free-floating in 2% paraformaldehyde. Approximately four to six sections distributed evenly within the area of the lesion (approximately −2.3 to −3.9 relative to Bregma) (Paxinos and Watson, 1986) were used for staining.

For immunocytochemistry, sections were placed in 4% paraformaldehyde for 5 min, rinsed twice in phosphate-buffered saline (PBS) and blocked in 10% goat serum/0.3% triton for 1 h at room temperature. Primary antibodies specific for cleaved forms of either caspase-3 or -9 (9661S, 9501S; Cell Signaling, Beverly, MA) were diluted 1:100 in PBS containing 2% goat serum/0.2% triton. Antibody 9661S detects only the 17–20-kDa fragment of caspase-3 in human, rat and mouse. Antibody

9501S detects only 37- and 17-kDa fragments of caspase-9 in human and rat. Neither antibody recognizes full-length forms of these caspases. Alternatively, some sections were incubated with SK398 (anti-cleaved caspase-3), SK440 (anti-cleaved caspase-8) (both from SmithKline Beecham), or Bur81 (anti-caspase-9) (Burnham Institute) at a dilution of 1:1000. Antibody SK398 is a neo-epitope peptide antibody generated to the C terminus of the p20 subunit of caspase-3 (GIETD). It detects only an 18-kDa fragment of caspase-3 in rodents. Antibody SK440 was raised against a p20/p10 fusion protein purified from *E. coli*. It recognizes epitopes in the p20 fragment of caspase-8. It does not react with endogenous caspase-8 expressed by Jurkat cells, or with recombinant full-length caspase-8–spiked Jurkat extracts (Velier et al., 1999). Bur81 reacts preferentially with 32–33- and 15-kDa fragments of caspase-9, but also detects the 48-kDa proform of this caspase, as shown in Figure 2 below. Sections were incubated in these solutions overnight (16 h/4°C). In some cases, mouse monoclonal antibodies against neuronal nuclear protein (NeuN) (Chemicon, Temecula, CA) or glial fibrillary acidic protein (GFAP) (Sigma, St. Louis, MO) were diluted at 1:100 in solution with caspase primary antibodies to serve as cell-type specific markers. After incubation, sections were rinsed twice (5 min each) in PBS (4°C) and then incubated with secondary antibodies (1:50) for 1 h (room temperature). Either goat anti-rabbit FITC (Sigma) or goat anti-mouse Texas Red (Accurate Chemicals, Westbury, NY) served as secondary antibodies. Finally, the sections were rinsed twice (5 min each), mounted, covered with anti-fade mounting medium, sealed with glass coverslips, and visualized with a confocal microscope. Confocal parameters were kept constant to allow valid comparisons between experimental groups. Method controls omitted either primary or secondary antibodies.

For sections co-labeled with TUNEL, Tdt reaction mix was applied for 1 h (37°C) after rinsing off primary antibodies. Tdt reaction mix consisted of dNTPs, Tdt buffer, and Tdt enzyme (Life Technologies, Rockville, MD) in sterile, ultrapure H_2O. The TUNEL reaction was stopped in 0.1 M EDTA, pH 8.0 for 2–5 min. Sections were rinsed twice in PBS (5 min each), and incubated with secondary antibody + DN-Avidin-FITC (Vector Labs, Burlingame, CA) for 1 h (room temperature). Thereafter, sections were rinsed, sealed with coverslips, and viewed with a Nikon TE300 inverted fluorescent microscope.

Neurological Evaluation

All neurological examinations were performed by an individual blinded to treatment. Complete methods for motor and cognitive testing have previously been described (McIntosh et al., 1989; Fox et al., 1998). Motor testing was performed at 7 and 14 days after trauma (McIntosh et al., 1989). Composite scores were derived from three separate motor tests: (1) flexion test—measures forelimb and paw (left and right) extension and placement in response to a perceived fall, (2) lateral pulsion test—measures body and leg resistance (left and right) to a lateral push, (3) inclined plane test—assesses the ability to maintain balance (in the vertical, left lateral and right lateral positions) on a progressively inclined surface. Scores for each limb on the individual tests range from 0 (maximal deficit) to 5 (no deficit). These were combined for a possible total maximal composite neuroscore of 35.

Morris water maze training began at day 14 after injury, and concluded after a total of 16 trials were completed in blocks of 4/day. For each trial, rats were gently placed into a tank of opaque water that had extramaze visual cues. The latency, or time required to locate a hidden, submerged, platform was recorded. After reaching the platform, animals were allowed to remain on it for 10 sec before they were removed, dried and kept in a warm environment until the next trial. Animals that failed

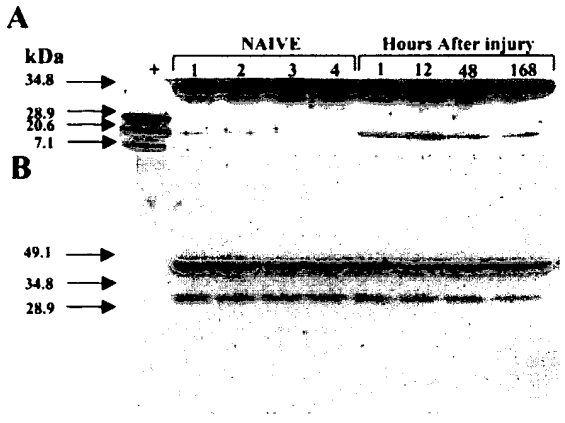

FIG. 1. Temporal expression of caspase-3 cleavage fragments in injured cortex after traumatic brain injury (TBI). Anesthetized rats (n = 5–6/group) were subjected to lateral fluid-percussion trauma (2.5–2.6 ATM). Injured cortex was analyzed by immunoblot at various times after injury (1–168 h). (**A**) A blot shows results from one animal at each time point, which is representative of results obtained from all animals in each group. The molecular weight of each cleavage fragment appears on the left. Bands corresponding to a 32-kDa proform and a 17-kDa cleavage fragment are present; expression of the 17-kDa fragment increased after trauma. Recombinant active caspase-3 was used as a positive control. Naive rats served as injury controls. (**B**) β-actin was directly assessed as an internal methods control. As β-actin may itself be cleaved by caspases, some control blots were overexposed, revealing a ~31 kDa cleaved fragment of β-actin which was unchanged by trauma.

to reach the platform within 2 min after placement in the tank, were gently placed on the platform for 10 sec before removal. Data are presented as the average latencies for the treated or untreated groups on each individual day of training.

Data Analysis

Densitometric immunoblot analysis data were assessed by ANOVA followed by post-hoc Dunnett's test. Motor neuroscores were analyzed by nonparametric Mann-Whitney *U* tests. Morris water maze data were analyzed by repeated measures ANOVA followed by multiple two-tailed *t* tests with Bonferroni (Dunn's) correction. A p value of <0.05 was considered statistically significant.

RESULTS

Immunoblot analysis of caspase-3 indicated the presence of a 32-kDa proform in all brain samples (Fig. 1A), but not in a recombinant active caspase-3–positive control. A 17-kDa cleavage fragment of caspase-3 was evident at 1, 12, and 48 h after injury. Blots were directly reprobed with β-actin antibody (without stripping) (Liao et al., 2000) which served as an internal control for protein loading and transfer. Loading and transfer of proteins was equal in all wells, as shown by equivalent signal intensity of a 42-kDa β-actin internal control (Fig. 1B; also see Fig. 3C below). However, because caspases are known to cleave β-actin, some control blots were overexposed to determine whether cleavage fragments of this protein were present. In some cases, a 30-kDa β-actin cleavage fragment was detected under this condition, but it was neither robust, nor trauma-dependent (Fig. 1B), thus verifying that β-actin was a suitable internal control.

Specificity studies showed that antibody Bur-81 did not react with either full-length or cleaved forms of caspases-3, -6, -7, -8, or -10, expressed by *in vitro* translation. Bur-81 preferentially reacted with the 32–33- or 15-kDa cleaved forms of caspase-9, but also detected the 48-kDa pro-form of this caspase (Fig. 2A). Similarly, the antibody detected the 48-kDa pro-form of caspase-9 in all brain extracts, as well as 32–33-kDa cleaved fragment (Fig. 2B). Expression of the 15-kDa cleavage fragment was low in naive controls, but increased after TBI. Densitometric analysis of the 15-kDa subunit revealed significant elevations over uninjured controls at 1 and 12 h after injury (Fig. 2C). The appearance of the 32–33-kDa cleaved fragment in both naive and injured cortex is consistent with observations that caspase-9 is autoprocessed in brain and peripheral nerve (Krajewski et al., 1999).

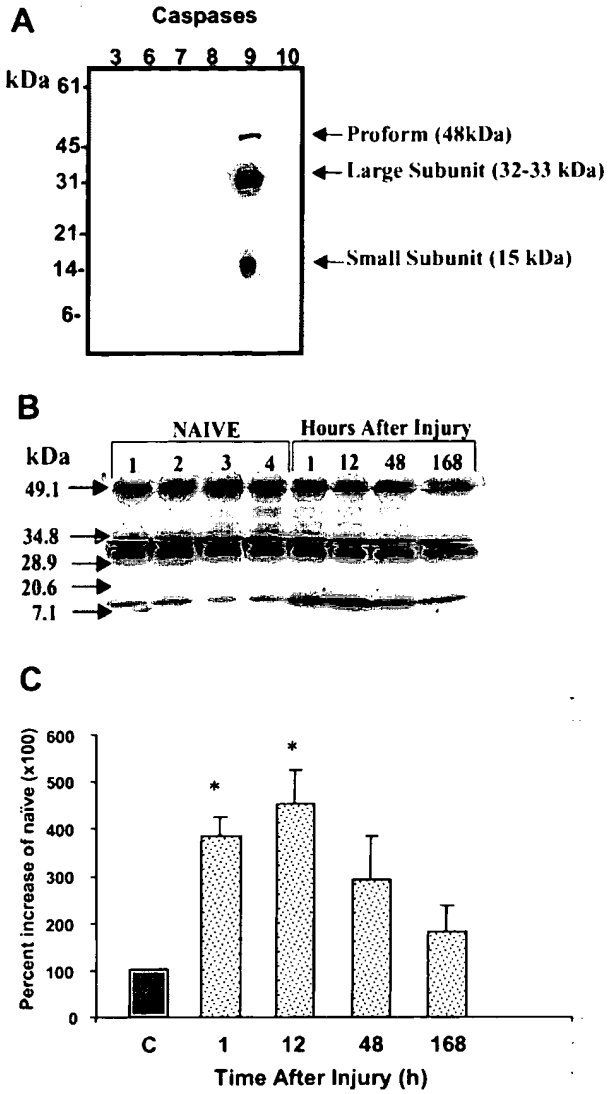

FIG. 2. Specificity of the Bur81 anti-caspase-9 antibody and temporal expression of caspase-9 cleavage fragments in cytosolic extracts of injured cortex after traumatic brain injury (TBI). (**A**) Immunoblots utilizing extracts from cells expressing *in vitro* translated caspases-3, -6, -7, -8, -9, and -10 show that Bur81 reacts primarily with the ~32–33 and 15-kDa processed (cleaved) forms of caspase-9, and also with the 48-kDa pro-caspase-9. (**B,C**) Rats (n = 5–6/group) were subjected to lateral fluid-percussion trauma, and the injured cortex assessed by immunoblotting with Bur81 as described for Fig. 1. A representative immunoblot is shown in B. Bands corresponding to a 48-kDa proform and 32–33- or 15-kDa cleavage fragments are present; expression of the 15-kDa fragment increased after trauma. A 32–33-kDa subunit of processed caspase-9 is known to be present in naive cortex and in peripheral nerve. (**C**) Immunoreactivity of the 15-kDa cleavage fragment is expressed as arbitrary densitometric units. Data were transformed to the percentage of densitometric levels from naive animals visualized on the same scanned blot. Bars indicate means ± SEM for each timepoint. $*p < 0.05$ versus controls by ANOVA and Dunnett's post-hoc test.

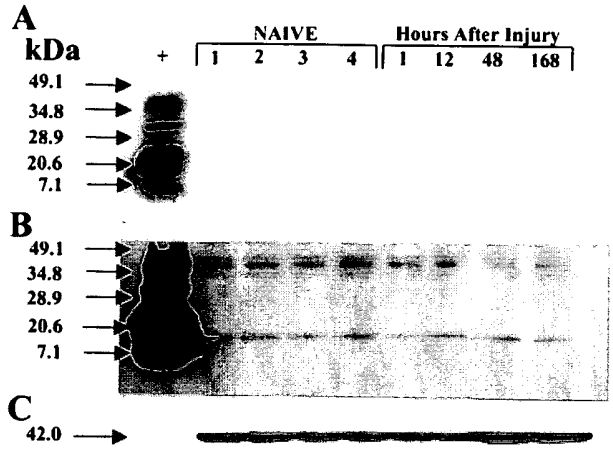

FIG. 3. Temporal expression of caspase-8 cleavage fragments in cytosolic extracts of injured cortex after traumatic brain injury (TBI). Rats (n = 5–6/group) were subjected to lateral fluid-percussion trauma, and the injured cortex assessed by immunoblotting as described for Fig. 1. (**A–C**) The blot shows results from one animal at each time point, which is representative of results obtained from all animals in each group. The molecular weight of each cleavage fragment appears on the left. In A, the blot was exposed for 15 sec to detect cleaved products present in the recombinant caspase-8 control, which undergoes autoprocessing. In B, the same blot was exposed for 8 min to determine whether caspase-8 cleavage fragments were present. At this exposure, bands corresponding to 38–40- or 14-kDa cleavage fragments were detected, although a p55-kDa proform was not. Expression of the 38–40- or 14-kDa cleavage fragments was unchanged by trauma. The β-actin internal control is shown in C.

There was no evidence for injury-induced cleavage of caspase-8, despite the fact that antibody SK440 detected several cleavage fragments in recombinant active caspase-8–positive control samples run on the same gels (Fig. 3A). Blots were subjected to multiple exposures, the longest of which revealed the presence of 38–40- and 14-kDa cleavage fragments in all brain samples, regardless of injury status (Fig. 3B).

To determine the distribution and cell-type expression patterns of caspases within the injured cortex and hippocampus we performed qualitative immunocytochemistry. Four to six sections distributed evenly within the extent of the lesion were chosen and stained for caspases-3, -9, or -8. For caspases-3 and -9, commercially available (Cell Signaling) antibodies, designated 9661S and 9501S, respectively, were used. The antibodies do not detect pro-forms of these caspases; they are specific for cleaved forms, and thus can be used as a cellular marker of caspase activation. Cleaved forms of caspases-3 and -9 were expressed mainly in the inner cortex along the border of the corpus callosum at 4 h after injury (Fig. 4), but were distributed widely throughout the entire region of injured cortex by 24 h after injury (Fig. 5). Active forms of caspases-3 and -9 were also expressed in the CA3 and CA2 regions of the hippocampus, particularly at 24 h after injury (Fig. 6). Selective cell loss was also evident in the CA3 region at this time.

In contrast to the relatively widespread distribution of active caspases-3 and -9 at 4 and 24 h after injury, expression of caspase-8 was limited to only a few cells within the injured cortex at either time (Fig. 7A). These

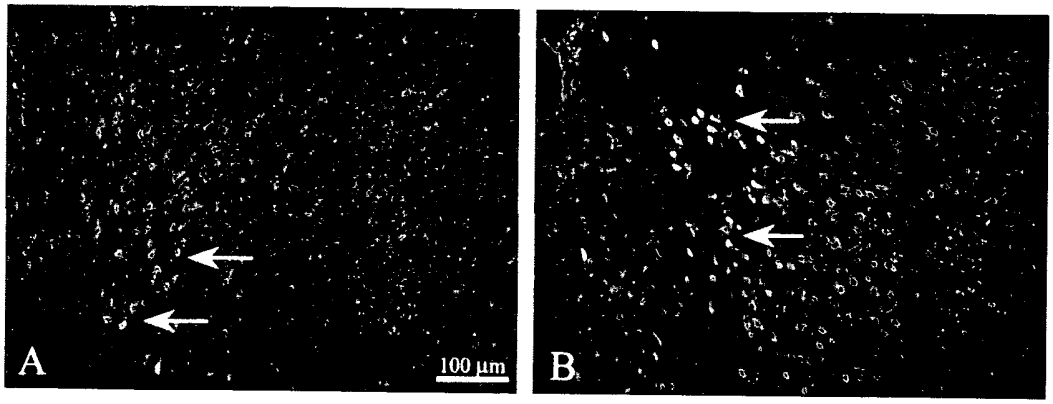

FIG. 4. Distribution of active caspase-3 (**A**) or caspase-9 (**B**) (antibodies 9661S or 9501S, respectively) in the inner cortex after lateral fluid-percussion injury, as visualized by fluorescent immunohistochemistry. Injury was produced as described in Fig. 1. At 4 or 24 h after injury, sham-injury brains were removed and stained as described in methods. Low-power images (×10) of the inner injured cortex at 4 h after injury are shown. Arrows indicate positively stained cells near the border of the corpus callosum. Images are representative of data obtained from n = 3 rats/group.

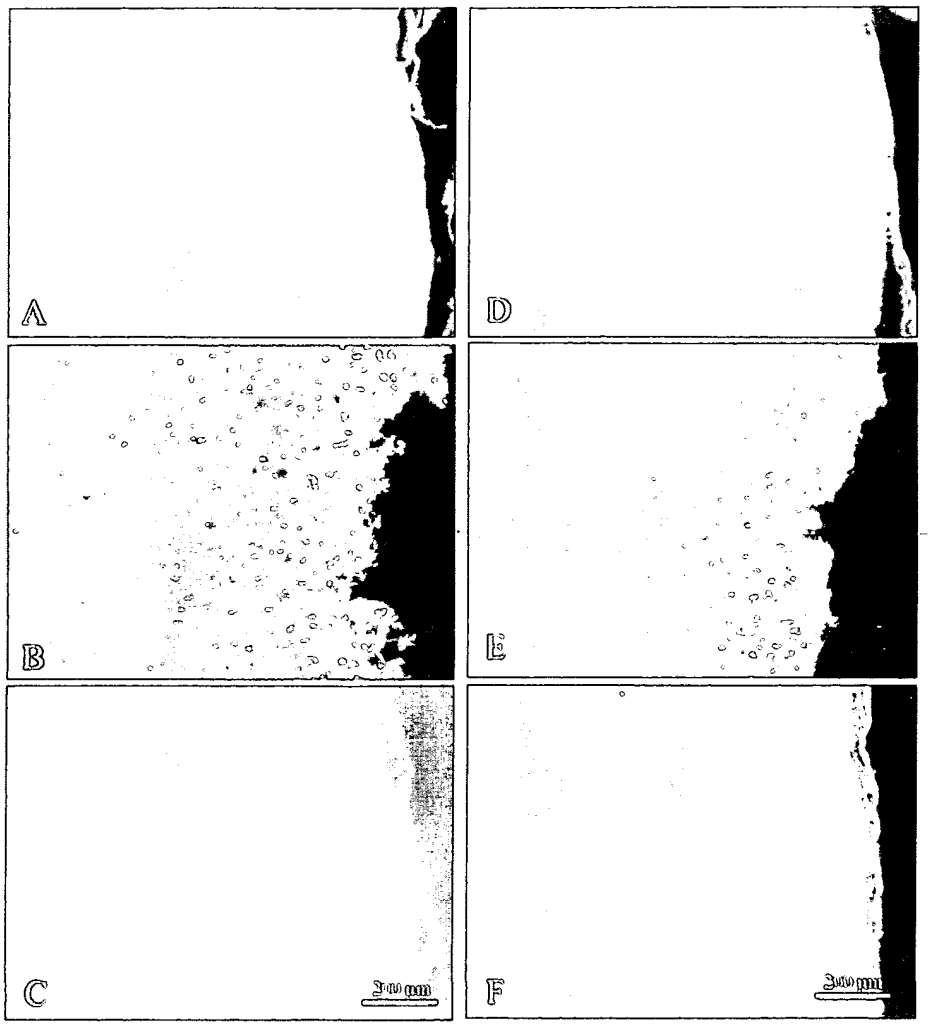

FIG. 5. Distribution of active caspase-3 (**A–C**) (antibody 9661S) or -9 (**D–F**) (antibody 9501S) in the outer cortex after lateral fluid-percussion injury. Data were obtained from the same rats described in Fig. 4. Low-power images (×10) of outer injured cortex were obtained at 4 h (A,D) and 24 h (B,E) after TBI, or 24 h after sham-TBI (C,F).

cells morphologically resembled neurons and glia. Even though few cells stained positively, such staining was associated with injury, as sham-injury controls showed no evidence of any positive staining (Fig. 7B).

Double-label immunocytochemistry revealed that cleaved caspases-3 and -9 were expressed by neurons, but not astrocytes of the injured cortex (Figs. 8 and 9). Staining in neurons was diffuse, cytosolic and present in the soma as well as what appeared to be dendrites, on some occasions (Figs. 5, 8, and 9). Caspase-9 and the cleaved fragment of caspase-3 were also colocalized to some extent with TUNEL-positive cortical cells that showed apoptotic-like condensed or punctate nuclear morphology (Fig. 10). However, this colocalization was not exclusive, as many TUNEL-positive and TUNEL-positive cells with apoptotic-like phenotypes did not express active subunits of either caspase, and some caspase positive cells were not TUNEL-positive.

To evaluate the effect of caspase inhibition on functional recovery after injury, animals were treated 15 min after injury with z-VAD-fmk, a pan-caspase inhibitor. This treatment significantly improved performance on a series of three separate motor tests administered 2 weeks later (Fig. 11A). In addition, in daily comparisons, z-VAD-fmk treated animals performed significantly better than controls in the Morris water maze test on days 2 and 4 of training (Fig. 11B). There was no significant difference in latencies on the first day of training, indicating that swim speeds were not significantly different between treated and control groups.

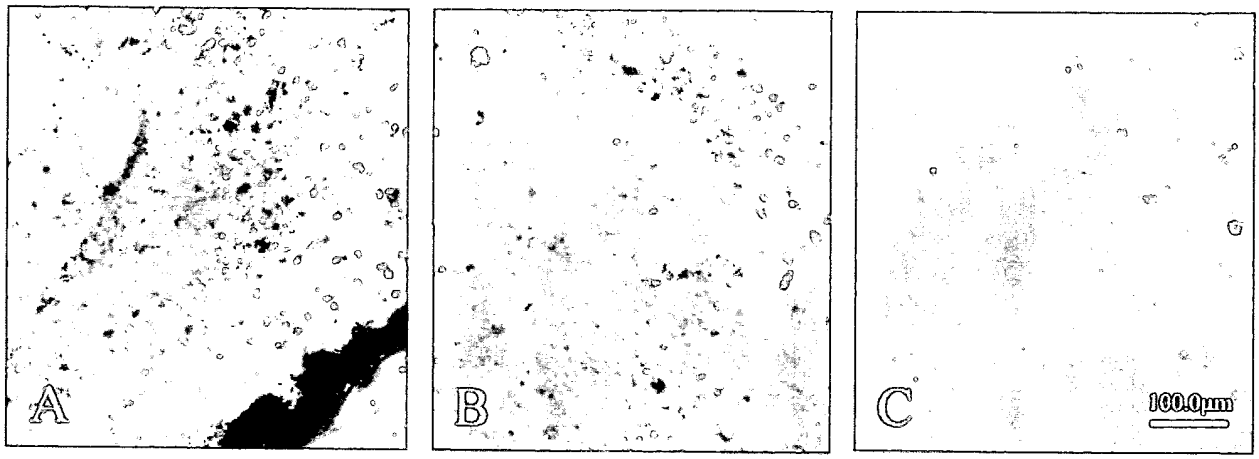

FIG. 6. Distribution of active caspase-3 (**A**) (9661S) or caspase-9 (**B**) (9501S) in the hippocampus 24 h after TBI. Many CA3 pyramidal cells stained positively for caspase-3 (A) or caspase-9 (B). Note that some cell loss is also visible in the CA3 region. The majority of CA2 cells at the junction with CA1 were caspase-3 or -9 positive, although most CA1 cells were not (data not shown). Few caspase-positive cells were observed in the hippocampus at 2 h after sham-TBI (**C**) or at 4 h after injury (data not shown).

DISCUSSION

Our studies show that caspases-3 and -9 are activated in injured cortex and hippocampus after traumatic injury. Cleavage products of these caspases were expressed throughout the cortex, primarily in neurons, and frequently co-localized with TUNEL-positive labeling. The latter associates these caspases with neuronal cell death that is likely to be, at least in part, apoptotic. In contrast, the presence of active caspase-8 was more difficult to detect than either caspase-3 or -9. Cleavage fragments for this caspase were not observed on immunoblots, and were only localized to a few cells by immunocytochemistry.

Caspase activation after brain injury has been assessed by a variety of methods, ranging from accumulation of degraded downstream substrates unique to a particular caspase (Pike et al., 1998; Beer et al., 2000b; Clark et al., 2000), to direct activity measurements utilizing oligopeptide substrates tagged with a fluorogen that produces an optical change upon cleavage (Yakovlev et al., 1997; Clark et al., 2000). In preliminary experiments, we found that, in the case of caspases-8 and -9, such fluorescence-based activity assays were nonspecific. In assays with Ac-LETD-afc (caspase-8) and Ac-LEHD-afc (caspase-9), the recognized substrate sequences for these caspases (Talanian et al., 1997; Thornberry et al., 1997), submicro-

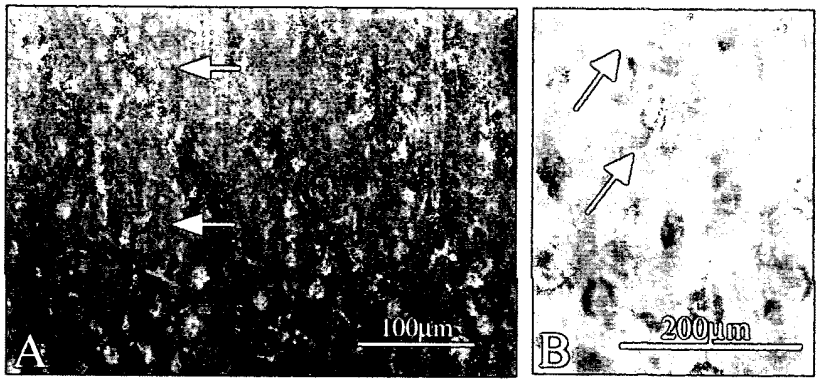

FIG. 7. Distribution of caspase-8 (antibody SK440) in the injured parietal cortex at 24 h after TBI. Low-power image shows that only a few cells distributed within the cortex expressed caspase-8 after injury (**A**; some positive cells indicated by arrows). Under higher power magnification, such cells resembled glia or neurons (**B**; cells indicated by arrows). Results are representative of $n = 3$ animals. Sections from 4 h after injury were also evaluated (including sections adjacent to those which showed positive staining for caspases-9 and -3), and showed a distribution of staining similar to that shown in A.

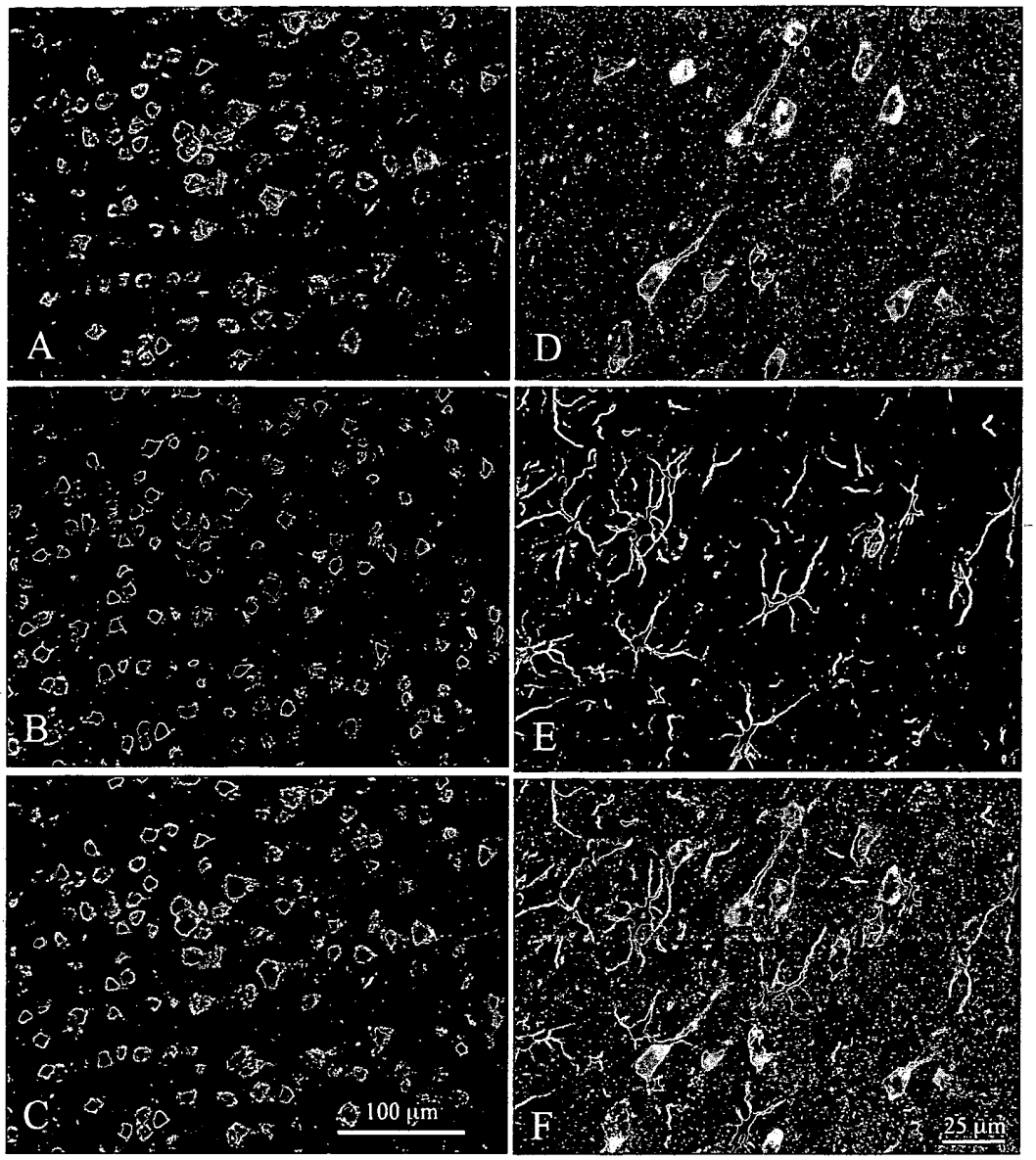

FIG. 8. Double-label immunocytochemistry for active caspase-3 (**A,D**) (antibody 9661S) combined with either NeuN, a neuronal marker (**B**) or GFAP (**E**), a marker for astrocytes. Staining for active caspase-3 was diffuse, cytosolic (**A**), and was localized to neuronal cell bodies (**B,C**), and in some instances, dendrites (**A**). Active caspase-3 was not frequently expressed by astrocytes (**E,F**). Images were taken from the injured parietal cortex at 24 h after TBI. They are representative of data from $n = 3$ animals.

molar concentrations of either recombinant caspase-3 or z-DEVD added to control (sham) samples respectively markedly increased or decreased apparent caspase-8 or -9 activity. Therefore, this method was replaced with detection of active caspases via immunoblot.

Our data indicated that caspases-3 and -9 were activated from 1 to at least 12 h after lateral fluid-percussion injury. These results are consistent with both the time-course for activation of caspase-3 we reported previously using a fluorogenic activity assay (Yakovlev et al., 1997), and with recently reported time-courses for caspases-3 or -9 utilizing immunoblotting methods (Beer et al., 2000b; Clark et al., 2000). Together, such findings suggest that cortical apoptosis occurs over an extended time-course, in agreement with studies which showed increased TUNEL-staining in the same model from 24 to 168 h after injury (Rink et al., 1995; Conti et al., 1998). These results are also consistent with the time course for acti-

vation of caspase-3 and apoptotic cell death reported in cortical contusion models (Yakovlev et al., 1997; Fox et al., 1998; Newcomb et al., 1999; Beer et al., 2000b; Clark et al., 2000).

In undisturbed neurons, the location of procaspase-9 is frequently mitochondrial, giving rise to a distinct organellar staining pattern (Krajewski et al., 1999). After ischemic injury, caspase-9 translocates from the mitochondria to the cytosol and nucleus, resulting in diffuse staining throughout the cell (Krajewski et al., 1999), which resembles that pattern we observed, suggesting that a similar translocation of caspase-9 occurs in both ischemic and traumatic injuries. Caspase-3 primarily resides in the cytosol (Krajewska et al., 1997), and thus staining for this caspase is usually diffuse, as seen here, and reported previously in other models of trauma (Beer et al., 2000b; Clark et al., 2000) or ischemia (Namura et al., 1998). Apart from visualization in the soma, we observed some expression of caspase-9 (and caspase-3) in what appeared to be dendrites. Dendritic expression of active caspase has been associated with synaptic loss and cell death due to excessive glutamate stimulation (Mattson et al., 1998). Thus, it is possible that caspase activation through this route could be a factor in traumatic injury. Expression of cytochrome c, calpain and active caspase-3 have also been observed in cortical axons, where they are hypothesized to contribute to neuronal demise in models of axonal injury (Buki et al., 2000). Together, these data suggest that activated caspases are not limited to somatic expression in injured neurons, and may reflect several different local routes through which neuronal death or synaptic reorganization could be initiated.

Qualitatively, the regional and cell-type distributions of active caspases-3 and -9 appeared similar, as they were evident in all layers of injured cortex, albeit somewhat preferentially in the inner cortex adjacent to the corpus callosum at 4 h after injury, but distributed throughout the region at 24 h after injury. Both caspases were expressed primarily by neurons rather than astrocytes, in agreement with findings from cortical contusion models, which also mainly located active caspase-3 expression to neurons trauma (Beer et al., 2000b; Clark et al., 2000). Caspase-3 appeared to be expressed in more cells than caspase-9 at both 4 and 24 h after injury. In this regard, it is worth noting that caspase-3 may be activated not only through extrinsic and intrinsic cascades, but also by additional factors, including granzyme B, caspase-11, and others, whose role in injury, if any, is uncertain (Yang et al., 1998; Kang et al., 2000).

We could not use antibodies 9661S or 9501S in the TUNEL double-label study, because these antibodies did not perform well on thin, mounted sections, but TUNEL-staining did not work well on thick free-floating sections. Therefore, antibodies SK398 and Bur81 were used in the TUNEL double-label experiments. In this regard, it is worth noting that SK398 is specific only for active caspase-3, whereas Bur81 preferentially stains cleaved fragments of caspase-9, but also reacts to a lesser extent with the pro-form of this caspase. Data from this experiment indicate that active caspase-3, and likely, active caspase-9, are expressed by either type I or type II TUNEL-labeled cells, but this association was not complete, as noted previously for caspase-3 by others (Beer et al., 2000b; Clark et al., 2000). This result may reflect the fact that active caspases are short-lived, and their temporal expression may not completely overlap the DNA fragmentation that gives rise to the TUNEL signal, but rather (and particularly in the case of caspase-9) precede it. Other factors may also contribute to the discrepancy. These would include observations that: some TUNEL positive cells are actually necrotic (Rink et al., 1995; de Torres et al., 1997); there may be a mixed apoptotic-necrotic phenotype (Allen et al., 1999); there is likely to be cross-talk between cell death mechanisms, and/or activation of certain apoptotic pathways may not be mediated by caspases (Susin et al., 2000; Thomas et al., 2000).

Presumptive involvement of the extrinsic apoptotic pathway in TBI is supported by evidence that Fas and Fas ligand are increased after TBI (Ertel et al., 1997; Beer et al., 2000a), as is TNF (Shohami et al., 1996; Knoblach et al., 1999). Yet, the present data show only limited caspase-8 activation. It is unlikely that our findings are due to antibody specificity issues. This antibody, which has previously been extensively characterized (Velier et al., 1999), strongly detected a positive caspase-8 control. In addition, we have utilized the antibody in an identical protocol to evaluate active caspase-8 expression after spinal cord injury (Huang et al., 2000), and others have used it in models of spinal ischemia and focal stroke (Velier et al., 1999; Matsushita et al., 2000). In all these studies, substantial expression of active caspase-8 was detected. More likely, the data reflect injury level or model issues. Caspase-8 activation was recently shown after more moderate fluid percussion injury (1.7–2.2 atm), where its temporal elevation correlated well with the time course of TNFα we observed after severe injury (2.6–2.7 atm) (Keane et al., 2001a). In our hands, more moderate injury (2.0 atm) did not consistently elevate TNFα. Thus, there are apparent differences in actual injury levels between laboratories, and it is possible that our severe injury level may be required to induce substantial caspase-8 activation. This is supported indirectly by a recent study in the cortical impact injury model, which demonstrated increases in both caspase-8 mRNA and activation, and in

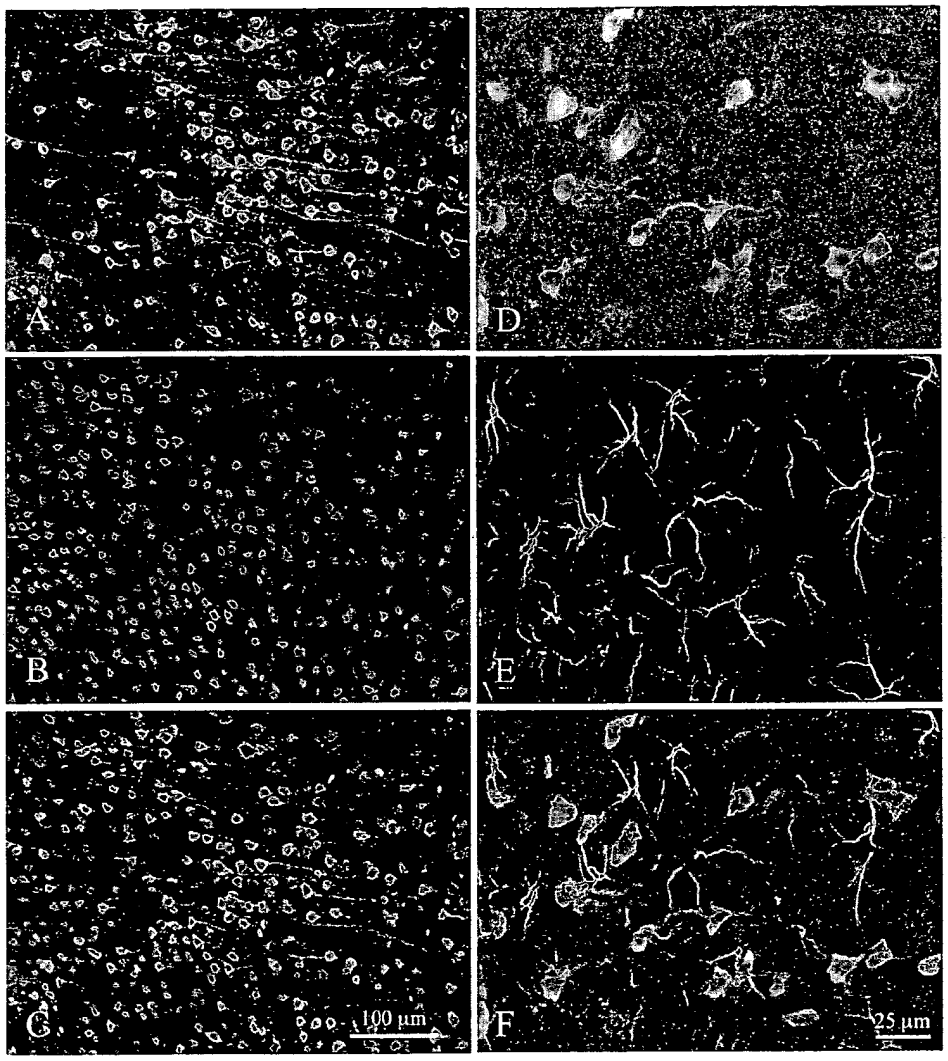

FIG. 9. Double-label immunocytochemistry for active caspase-9 (**A,D**) (antibody 9501S) combined with either NeuN, a neuronal marker (**B**) or GFAP (**E**), a marker for astrocytes. As with caspase-3, staining was diffuse, cytosolic and was localized to neuronal bodies and in some instances, dendrites (**A,D,C**). Active caspase-9 was not frequently expressed by astrocytes (**F**). Images were taken from the injured parietal cortex 24 h after TBI. They are representative of data from $n = 3$ animals.

addition, associated caspase-8 with neurons and glia (Beer et al., 2001). This injury model is known to invoke an ischemic response that may only be present at severe levels of fluid percussion injury (Bryan et al., 1995).

Our caspase-9 data are the first to show the cortical distribution of this caspase after injury, as well as to evaluate its expression in specific cell-types, or in conjunction with TUNEL staining. The results are consistent with reports of increased expression of apaf-1 and cytochrome c after trauma (Yakovlev et al., 2001), as well as alterations of endogenous inhibitors of this pathway, such as bcl-2 or XIAP (Clark et al., 1999; Lu et al., 2000; Keane et al., 2001a,b). Together, these data collectively provide strong support for the intrinsic pathway of apoptotic cell death after TBI.

Treatment with the pan-caspase inhibitor z-VAD-fmk improved motor and cognitive function, compared to vehicle controls. This likely relates to caspase expression both in cortex and hippocampus, as shown here for caspase-9 and previously for caspase-3 (Yakovlev et al., 1997). Z-VAD-fmk was also neuroprotective in a mouse model of controlled cortical impact, where it reduced lesion volume (Fink et al., 1999), and in mouse and rat models of ischemia, where it both reduced lesion volume and improved neurological function (Loddick et al., 1996; Hara et al., 1997; Fink et al., 1998; Ma et al., 1998).

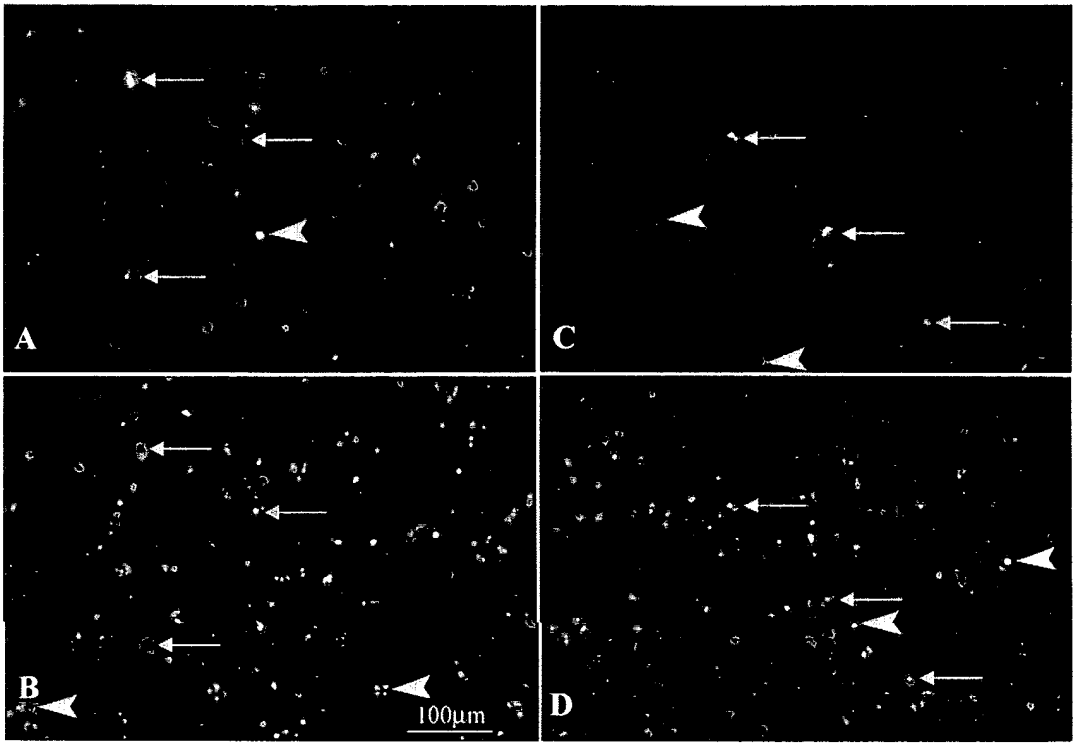

FIG. 10. Colocalization of caspases and TUNEL staining in rat cortex at 24 h after TBI. Fluorescent immunostaining was used to detect either active caspase-3 (SK398 antibody) (**A**) or caspase-9 (Bur81 antibody) (**C**) as indicated in representative fields (×40). (**B,D**) Images show TUNEL-positive staining observed in the same fields as A and C, respectively. Arrows denote cells positive for caspase as well as TUNEL labeling. Chevrons denote cells positive for either caspase or TUNEL labeling, but not both.

Z-VAD-fmk is an irreversible inhibitor of most caspases, showing greatest inhibition of caspases-1, -5, -8, and -9, but also significant inhibition of caspases-3, -4, -6, and -7 (Thornberry et al., 1997). Of these, caspases-4, -5, -6, and -7 have not yet been associated with TBI, and may not even be present in the CNS (Eldadah and Faden, 2000). However, we previously observed improved neurological recovery concurrent with reductions in TUNEL-labeled cells and DNA laddering after selective inhibition of caspase-3 with z-DEVD-fmk in our model (Yakovlev et al., 1997), implicating this caspase in tissue damage and functional deterioration. Similar findings were reported after caspase-3 inhibition in a model of controlled cortical impact, although behavioral status was unchanged (Clark et al., 2000). The parenchymal injection scheme employed in the latter study may have led to local tissue preservation, but prevented diffusion of z-DEVD-fmk to brain regions (cortex, hippocampus) that are represented in behavioral outcome measures. Caspase-1 has also been implicated in TBI, but here again, the data are mixed. The selective caspase-1 inhibitor z-YVAD-fmk reduced lesion volume in a mouse cortical contusion model, and dominant-negative caspase-1 mutant mice showed improved neurological function and reduced lesion volumes (Fink et al., 1999). However, activation of caspase-1 was not significantly elevated after lateral fluid-percussion injury, though mRNA for the proform did increase (Yakovlev et al., 1997). Nonetheless, caspase-1 activation has been observed after clinical head injury (Clark et al., 1999). Whether such discrepancies reflect potential ischemic/metabolic complications associated with clinical head injury and/or model specific anomalies remains to be determined. Specific proof of an important role for caspases-8 or -9 awaits additional studies, perhaps with ribozyme constructs, given issues with the specificity of currently available selective inhibitors for these caspases.

In addition to inhibition of caspases, z-VAD-fmk was recently found to inhibit calpains (Wolf et al., 1999; Blomgren et al., 2001), enzymes which are independently recognized as important mediators of necrotic cell death, tissue loss and dysfunction in both traumatic and ischemic injuries (Kampfl et al., 1996; Saatman et al., 1996; Pike et al., 1998; McCracken et al., 1999; Zhao et al., 1999; Buki et al., 2000). The IC50 of z-VAD is actually lower for m- and u-calpain (15 μM) than for caspase-3

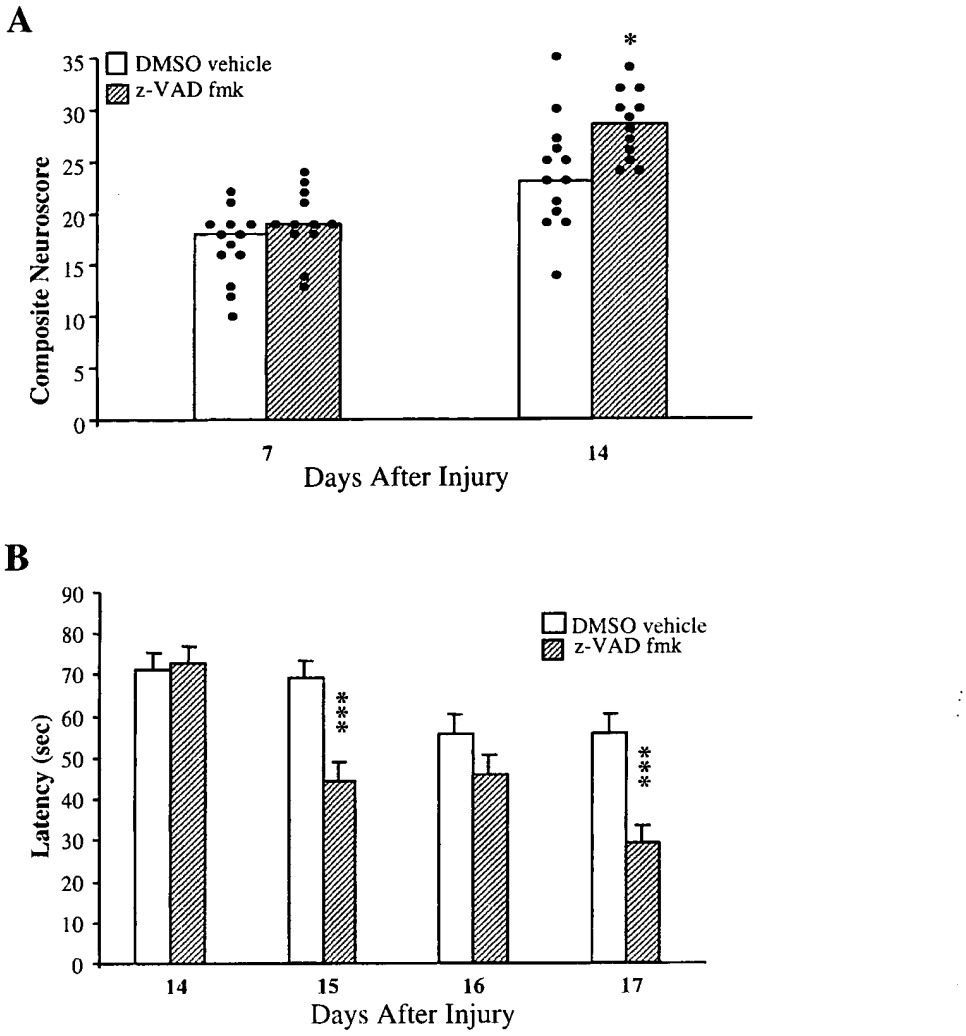

FIG. 11. Pan-caspase inhibitor z-VAD-fmk improves neurological recovery after traumatic brain injury. Z-VAD-fmk (25 mM in 5 μL), was injected intracerebroventricularly 15 min after lateral fluid-percussion–induced brain injury. (**A**) Bars represent median combined score on three separate tests of motor function (flexion, pulsion, and inclined plane). Dots represent scores of individual animals (z-VAD-fmk, $n = 12$; vehicle [DMSO], $n = 13$). The best possible combined score is 35. Animals treated with z-VAD-fmk scored significantly better than DMSO controls 14 days after injury. $^{*}p < 0.05$ versus vehicle by Mann-Whitney U comparison. (**B**) Bars represent the daily mean ± SEM of latency to find a hidden platform for each group over four trials of a Morris water maze visuospatial learning paradigm. Four consecutive days of training (for a total of 16 trials) commenced 14 days after traumatic brain injury. Data are from the same animals/experiment shown in A. Z-VAD-fmk–treated animals performed significantly better on days 2 and 4 of water maze testing than did vehicle (DMSO)–treated controls. $^{***}p < 0.001$ for z-VAD-fmk versus vehicle from the same day. Maze data were analyzed by repeated measures ANOVA followed by post-hoc t tests with Bonferroni correction.

(60 μM) (Blomgren et al., 2001); thus, it is possible that some of the beneficial effects of z-VAD-fmk may reflect inhibition of calpains. From a treatment perspective, an agent which inhibits both calpains and caspases may be optimal for clinical use, as it would target both necrotic and apoptotic cell death. This may be particularly important, since evidence suggests that prevention of either necrosis or apoptosis may shunt the injury response toward the alternate death pathway (Pohl et al., 1999; Lewen et al., 2001; Susin et al., 1998). In addition, calpain- and caspase-mediated cell death pathways appear to share points of intersection as well as common features (reviewed in Wang, 2000). Illustrative examples include that the endogenous calpain inhibitor calpastatin is degraded by active caspases (Wang et al., 1998a), and that both enzymes degrade spectrin, a major component of cytoskeleton (Wang et al., 1998b).

In summary, we show evidence for the activation of

several caspases after TBI. Activation of caspases-3 and -9 was significantly elevated from hours to days after injury, in contrast to active caspase-8, which was expressed in only a few cells. In addition, the pan-caspase inhibitor z-VAD-fmk improved motor and cognitive neurological dysfunction after TBI. Together, these data support an involvement of several caspases in secondary cell death and neurological dysfunction after TBI, and suggest that anti-caspase treatment strategies may be potentially useful after traumatic brain injury in humans.

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